THE ENZYMATIC HYDROLYSIS OF GLUTAMINE AND GLUTATHIONE BY GLUTAMINASE

by

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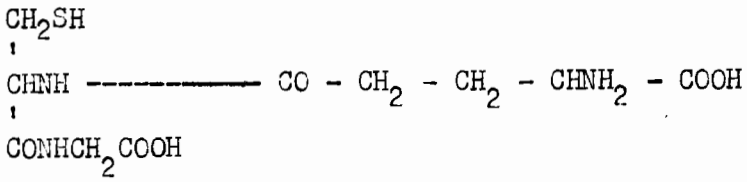
TABLE OF CONTENTS

INTRODUCTION

Hopkins (17) first described the substance known as "glutathione", which in its reduced form is a tripeptide of glycine, cysteine, and glutamic acid.



Very little is known about the functions of glutathione, although the high concentration and ubiquitousness of this tripeptide in the blood and tissues of animals has brought it to the attention of many workers. Barron proposed that glutathione maintained enzymes active by reducing their sulfhydryl groups. Hopkins believed that glutathione acted as a reversible oxidation reduction system for which ascorbic acid might be a coenzyme. Harrow suggested that glutathione might be the source of the amino acids used in detoxication. None of the ascribed roles, however, account for the wide distribution of glutathione, and at present, there is little conclusive evidence as to how glutathione does function in the body mechanism.

The development in 1948 by Nakamura and Binkley (25) of an adequate method for the identification of the products of enzymatic hydrolysis has made possible the study of the nature and mechanism involved. That same year Binkley and Nakamura reported that the enzyme responsible for the

first step in the hydrolysis of this tripeptide, the cleavage of the gamma glutamyl bond, was abundantly and almost exclusively found in the tissues of the kidney. Data identifying this enzyme as the same enzyme which hydrolyzes glutamine to glutamic acid and ammonia is presented here. This enzyme was first described in 1935 by Krebs (20), who designated it "glutaminase".

The work to be presented here has been thought to suggest four things: first, that glutaminase acts as a cation exchange resin in the tubules of the kidney; second, that glutathione serves as a constant source of glutamine for this enzyme; third, that glutathione serves as a transport mechanism carrying certain materials to the kidney for excretion; and fourth, that from observations of the effect of ions on this enzyme, glutaminase plays an important role in reabsorption mechanism of the tubules of the kidney.

The theory and interpretation of data presented here must be attributed largely to the foresight of Dr. Francis Binkley.

HISTORICAL

I. Enzymatic Hydrolysis of Glutathione by Animal Tissues

The isolation in 1921 of glutathione by Hopkins (17) from yeast, muscle, and liver opened a research area which has since been the source of much investigation and much speculation. Though Hopkins mistakenly believed this compound to be a dipeptide of glutamic acid and cysteine, he was not mistaken in his opinion concerning its abundance in animal tissues. Nine years later, Kendall and coworkers (18 and 19) published two papers containing the first searching study of glutathione. It was these workers who correctly described the structure of this compound as a gamma glutamyl cysteinyglycine peptide, although they were mistaken as to the opinion that glutathione was hydrolyzed by erepsin into its constituent amino acids. In the same year, Harington and Mead (16) achieved its synthesis, and thus made it available to researchers.

In 1932, the German worker, Lohmann (22) was the first to describe a biological system in which glutathione played a role. He found reduced glutathione to be a specific activator of the enzyme, glyoxalase, which converts methyl glyoxal to lactic acid. Woodward (34), in 1935, described glyoxalase as a reagent for the microdetermination of glutathione, and further described the activation of glyoxalase, finding it dependent within a certain range upon the concentration of glutathione.

It was Woodward, et al (34), in their studies of glyoxalase, whose experiments first demonstrated that glutathione was hydrolyzed enzymatically

by tissues of the animal. These workers were able to show the presence of a powerful inhibitor of the enzyme glyoxalase in the kidney of the rat. Since it exhibited a number of properties indicative of an enzyme, and since with incubation of glutathione and kidney antiglyoxalase in solutions an increase of titratable carboxyl groups was detected which stopped at a value corresponding closely to the theoretical-hydrolysis of one peptide linkage,they described the antiglyoxalase as an enzyme hydrolyzing glutathione into fragments which were not able to activate glyoxalase. They further reported that the rate of increase of titratable carboxyl groups was proportional to the rate at which glutathione lost its ability to activate glyoxalase.

Incubation of oxidized glutathione with kidney extracts showed a considerably higher rate of hydrolysis than that found with the reduced form. Here again, the increase in carboxyl groups was correlated with the theoretical value for the hydrolysis of two peptide linkages of the oxidized glutathione molecule. The pH optimum of 7, reported in this paper by Woodward, has since been observed by many workers.

Schroeder and coworkers (28) concluded that the products of hydrolysis of glutathione were glycine and glutamylcysteine,from their study of the enzymatic nature of the kidney antiglyoxalase. Later, however, Schroeder and Woodward (30),working with various kidney preparations, revised their original conclusion. When synthetically prepared samples of the glutamyl-cysteine to which they had ascribed the color obtained by Sullivan's (31) method were tested, no color developed, but Sullivan determinations on samples of the digested kidney-glutathione mixtures showed a nearly

theoretical amount of cysteine was being formed, and their titration values corresponded more closely to the theoretical cleavage of two peptide linkages. In 1935 they reported that cysteinylglycine and glutamic acid were the products of the hydrolysis of glutathione by kidney extracts. Simultaneously, Grassmann, et al (12), were studying the enzymic cleavage of glutathione. These workers found glutathione in its oxidized or reduced form was not attacked by proteases, pepsin, pancreatic proteases, or papain. No hydrolytic action of the dipeptidase and amino peptidase from yeast and intestine was demonstrated against glutathione. A rapid cleavage of oxidized glutathione, however, was found with the carboxypolypeptidase of the pancreas, although hydrolysis stopped after half of the peptide linkage in the molecule had been disrupted. They attributed the failure of the carboxypeptidase to attack the reduced form of glutathione to the sulfhydryl group of the tripeptide. From filtrates of the unhydrolyzed material, they isolated glycine in more than an eighty per cent yield as the ester hydrochloride.

Mason (23), repeating the earlier work of Kendall and his group, who found glutathione apparently hydrolyzed into its constituent amino acids when incubated with erepsin at 37°for 2 to 5 days, showed that under the same conditions, but without erepsin, pyrrolidonecarboxylic acid was split off, and the remaining cysteinylglycine then hydrolyzed by erepsin. Erepsin did not hydrolyze both peptide bonds of glutathione.

In 1942, Woodward and Reinhart (35) presented evidence that pyrrolidonecarboxylic acid, as well as glutamic acid, is formed by the enzymatic hydrolysis of the gamma glutamyl bond. The splitting of this bond results in the formation of different proportions of glutamic acid

and pyrrolidonecarboxylic acid, depending upon the pH of the digestion mixture. Thus, at pH's below 6, glutamic acid was found to predominate, but with increased alkalinity, the ratio of pyrrolidonecarboxylic acid increased.

More recently, Neubeck and Smythe (26) have demonstrated the presence of an enzyme in the liver of the guinea pig, lamb, rat, rabbit, beef and human, which is capable of hydrolyzing glutathione. To determine the total reducing groups in filtered preparations of the liver, they measured the amount of iodine uptake. A rapid increase between the time of killing the animal and the addition of the meta phosphate was shown. The increased uptake during this period they related to the cysteine being released. Experimentally, they observed the end point changed during the increase in titration from a definite point, that is characteristic of glutathione, to one that was not as definite, and more characteristic of cysteine. Comparative results with Sullivan determinations supported the belief that cysteine was being released during this period.

The enzyme described by Neubeck and Smythe is probably the same enzyme earlier described by Woodward and Reinhart (34). Reportedly it had an alkaline pH optimum between 8.5 and 9.0, and was comparatively insoluble, although usable solutions were obtained. Dialysis inactivated the enzyme, which could be reactivated by addition of the dialyzate, and extracts from other species reactivated the dialyzed enzyme. The coenzyme, found in high concentrations in the liver, was relatively stable to heat, and retained the ability to activate the dialyzed enzyme.

They reported that cysteinylglycine was not formed in the

hydrolysates, but their determinations were made on filtrates of digestion mixtures which were incubated for twenty-one hours at 25 C. It is impossible to assume that cysteinylglycine was not an intermediate in hydrolysis, in view of the period of incubation.

Until 1948, studies on the hydrolysis of glutathione by animal tissues had been handicapped by the lack of adequate methods for the identification of the products of hydrolysis. Although evidence had been presented that glutathione was hydrolyzed into its constituent amino acids, the mechanism involved in this breakdown had not been clearly described. The development of a specific method for the estimation of cysteine by Nakamura and Binkley (25), made possible the elucidation of the nature and mechanism involved in the complete enzymatic hydrolysis of glutathione. Sullivan's method measures cysteinylglycine plus cysteine, which allowed no quantitative or qualitative evidence of the second stage of hydrolysis.

By this method, Binkley and Nakamura (4) were able to show that the hydrolysis of glutathione is a two step process involving the intermediate formation of cysteinylglycine. Neubeck and Smythe had correctly concluded that gamma glutamylcysteine was not an intermediate in the hydrolysis. Schroeder and Woodward, and Woodward and Reinhart had correctly reported that the first step is the cleavage of the gamma glutamylcysteine linkage, but their results showing the production of cysteine were not valid.

Binkley and Nakamura found the enzyme responsible for the hydrolysis to cysteinylglycine in the rat limited to kidney tissues, but the enzyme

hydrolyzing the dipeptide was apparently found in all tissues. The enzyme involved in the cleavage of the gamma glutamylcysteine linkage was found to be more stable to heat and to be more active at lower pH values than the enzyme hydrolyzing cysteinylglycine. These results disagree with the earlier report by Neubeck and Smythe, that the enzyme of rat liver, referred to as a coenzyme, was stable to heat, and they found no evidence for the occurrence of a dialyzable component.

Binkley and Nakamura pointed out that glutathione is one of the few naturally occuring peptides which is well characterized and readily obtainable, and as such, should serve as a suitable model for studies of peptide bond formation. These workers realized that further studies of the products of hydrolysis and the mechanism of hydrolysis of glutathione might well yield information on the functions of this tripeptide found so abundantly in animal tissues.

II. Enzymatic Hydrolysis of Glutamine by Animal Tissues.

In 1935, Krebs (20) first described the enzymatic hydrolysis of glutamine into glutamic acid and ammonia by animal tissues. Hunter and Geddes (17) in 1938, and Grassman and Mayr (13) in 1933 observed in yeast extracts the enzymatic hydrolysis of glutamine while studying the specificity of asparaginase, and were unable to reach a conclusion as to whether glutamine and asparagine were split by one and the same enzyme. By comparison of the ratio of activity of asparaginase to the activity of glutaminase in certain tissues, Krebs showed that there is a specific "glutaminase" of the kidney, liver, brain, and retina

of animals.

Most of the properties of glutaminase known up to the present were described by Krebs in 1935, and the problems he recognized then, and the interpretations presented therein of his data, are primarily still valid. In 1932, Chibnall and Westall (6) reported that glutamine, heated (100°) in neutral solution, yielded pyrrolidonecarboxylic acid and ammonia. In this case, one amide and one amino group disappear for the one equivalent of ammonia formed. With Van Slyke's determination of amino nitrogen, Krebs calculated that with the disappearance of one amide group, one equivalent of ammonia was formed by the action of glutaminase. Thus enzymic splitting and neutral heat hydrolysis of glutamine yield different products.

In describing inhibitors, a peculiar phenomenon in the course of hydrolysis of glutamine by tissue extracts was observed. The hydrolysis originally has a high velocity, but the rate rapidly falls off. Krebs was aware that this decrease of activity was not due to the destruction of the enzyme, since fresh extracts did not restore the activity, but ascribed the inhibition to the glutamic acid formed in the hydrolysis. This inhibition is not due to thermodynamic equilibrium between glutamine and ammonium glutamate: the equilibrium of the reaction in physiological solutions lies at practically complete hydrolysis of the amide. He found the inhibition by glutamic acid to be competitive for the enzyme, and calculated that if the ratio of concentration in solution of glutamine to glutamic acid is 4:1, the enzyme is equally distributed between the two substrates. Mistakenly, he believed that

the inhibition by glutamic acid was specific, and reported that glutathione had no effect on the system.

In working with various tissue extracts, Krebs was convinced of the existence of different glutaminases ("brain type" and "liver type") distinguishable by their pH optima and their inhibition by glutamic acid. The observations that: (1) the pH curves for kidney, liver and brain glutaminase between pH 7 and 8, and of liver glutaminase between 8 and 9; (2) the inhibition of glutaminase by glutamic acid was characteristic of the enzyme, and yet this inhibition was not found in all tissue extracts; (3) rat kidney seemed to contain both glutaminases, the splitting of glutamine was partially inhibited by the presence of glutamic acid, and the pH curves showed two maxima, led Krebs to the decision that there were different glutaminases in the various tissue extracts.

From incubation experiments with retina extracts, Krebs found a deficit in the amount of amide nitrogen formed in comparison with the amount of ammonia which disappeared, which he thought indicated that the tissue utilizes glutamine in other ways than splitting it into glutamic acid and ammonia. Thus there appeared to be a cycle of ammonia in nervous tissue in which the conversion of ammonium glutamate into glutamine was but one step.

Krebs found support for his belief that the glutaminases were concerned in both the synthesis and the hydrolysis of glutamine in kidney, brain, and retina tissues from inhibition studies of "energy-giving reactions". He felt that the system, which synthesized glutamine,

consisted of glutaminase and an additional factor which is concerned with the transmission of energy. The thermodynamic equilibrium between ammonium glutamate and glutamine is changed by the transmission of energy and favors glutamine, and with this change, the enzyme, glutaminase, catalyses the attainment of this equilibrium. What the physiological function of the glutamine-glutaminase system is, Krebs did not even suggest.

In 1944, Archibald (1), investigating the physiological role of glutamine, described an enzymatic method for the determination of glutamine, using kidney extracts, which measured the ammonia formed by the action of glutaminase. The method has the advantage of being more specific in the presence of urea and asparagine than methods involving acid hydrolysis, and within limitations can be used for the determination of free glutamine in enzymatic hydrolysates of proteins. However, without further purification, the kidney extract liberates ammonia from adenosine and its derivatives in the tissues.

In studying the properties of the glutaminase preparation, he found that the addition of potassium cyanide to a concentration of 0.0025 M in digestion mixtures decreases the liberation of ammonia from asparagine and from other amino acids through the action of alpha deaminase. All attempts to purify the enzyme were found to be at the expense of the bulk of the activity, from which he correctly assumed a large, highly insoluble enzyme. Archibald agreed with Krebs that there might well be more than one glutaminase. He found, in agreement with Krebs, that the pH optima of kidney preparations was between 7 and 8.

By use of the enzymatic method of determination, Archibald was the first to report that blood plasma was without glutaminase, and the first to report finding glutaminase present in the kidneys of human beings. He added to the list of inhibitors of the enzyme the compounds p-benzoquinone, bromosulfalein, and atabrine.

Leuthardt and Glasson (21), in 1942, as a result of in vitro studies of tissue slices, suggested that glutamine can function as an ammonia donator and acceptor. The following year, experiments by Van Slyke, et al (36), on dogs in which urinary ammonia excretion was increased by administration of hydrochloric acid, pointed out four things: first, that the ammonia concentration is greater in the renal venous blood than in arterial blood; therefore, the ammonia excreted in the urine is not from preformed blood ammonia, but must be formed in the kidneys; second, that urea removed from the blood by the kidney was excreted unchanged: hence, urea was not the source of urinary ammonia; third, that the total ammount of alpha-amino acid nitrogen extracted from the blood by the kidneys was found to be too little to provide ammonia at the rate excreted: and fourth, that the amide nitrogen of glutamine was found to be less in the renal venous blood plasma than in the plasma of arterial blood. Thus it appeared that the role of the glutamine-glutaminase system of the kidney was to supply and control the secretion of urinary ammonia.

In 1945, Hamilton (15) drew attention to the instability of glutamine in the presence of such anions as phosphate, and the method of determination he developed was sensitized in its specificity for

glutamine in blood and tissues by removing interfering ions and substances such as ascorbic acid and glutathione. By Hamilton's method, glutamine reacts with ninhydrin, liberating carbon dioxide equivalent to the alpha amino nitrogen, and has the advantage over the enzymatic method that asparagine and adenosine and its derivitives are not affected, which permits measurement of glutamine in animal tissues. Hamilton reported that the normal concentration of glutamine in dog and human plasma varied from 6 to 12 mgs. per 100cc, and constituted 18 to 25 per cent of the total free amino acid carboxyl nitrogen of the filtrates. In reporting the distribution of glutamine in animal tissues, he found, as would have been expected, that the concentration of glutamine varied inversely with the concentration of glutaminase. Thus, glutamine is almost absent from the kidney, and is found in concentrations up to 22 mgs. of glutamine amide nitrogen per 100 g. of tissue in the heart.

McIlwain (24) investigated ammonia formation from glutamine by hemolytic streptococci, and reported that non-proliferating suspensions of this bacterium convert L-glutamine to L-glutamic acid plus ammonia. The action of this glutaminase was observed, however, only during glycolysis, and the rate of hydrolysis was of the same order as the assimulation of glutamic acid by growing organisms. McIlwain suggested that the acceleration of growth produced by the addition of glutamine, was, in fact an ammonia transfer.

In 1947, Greenstein, et al, (5),(7) (8) (10) (11) (27) published six papers on (1) the capacity of rat liver extracts to desamidate glutamine and

asparagine, and (2) the effects of pyruvate and phosphate on this desamination. From their data it appears probable that the effect of pyruvate on the desamidation of glutamine and asparagine is not concerned with glutaminase and asparaginase activity. In the presence of pyruvate at temperatures which completely inactivate glutaminase and asparaginase, the liver extracts retained the capacity to desaminate glutamine and asparagine. The relatively high activity of the pyruvate effect at pH 6.0, where glutaminase activity is nil, suggests the involvement of different enzymatic mechanisms. These workers suggested the probability that desamidation of glutamine and asparagine in the presence of pyruvate is affected by the formation of a labile intermediate compound between the amino acid amide and pyruvate. Carter and Greenstein (5) suggested that the most probable type of intermediate would be a dehydropeptide. According to this explanation, two enzymatic steps are involved, first, a condensation between the amide and carbonyl group of the keto acid to form a dehydropeptide; second, the splitting of the dehydropeptide to yield the corresponding amino acid, ammonia, and pyruvate. Attempts to isolate the intermediate dehydropeptide, or to recognize it during the course of the reaction, have as yet been unsuccessful. This mechanism seems highly improbable.

Both pyruvate and phosphate increase the rate of desamidation of glutamine in liver extracts. Phosphate has no effect on the desamidation of asparagine. The observed activation of glutaminase by phosphate followed the pH range of activity, the acid and heat

stability curves of glutaminase in the rat liver extracts. With these facts and in consideration of the high acceleration of desamination by this anion, a rate far greater than that of pyruvate at equivalent concentrations, the authors suggested that the effect of phosphate in liver extracts was a direct activation of glutaminase.

EXPERIMENTAL

Preparation of the Enzyme. Hog kidneys frozen under carbon dioxide were homogenized in a Waring Blendor with five volumes of 0.1 M Veronal for a period of 2 to 3 minutes. The homogenate was held at 50° for 15 minutes, cooled rapidly to 3°, and let stand overnight. The homogenate, after passage through cheese cloth, was centrifuged twenty minutes at 2500 rpm. After overnight dialysis against distilled water, equal volumes of the supernatant were mixed with acetate buffer pH 4.4, and centrifuged immediately for 15 minutes at 2500 rpm. All steps were carried out at 3°. The fraction precipitated was redissolved, and brought back to original volume with 0.1 M Veronal.

Further purification was possible by the addition of ethanol at 3°. The fraction precipitated up to 0.6 volumes of ethanol was saved, and brought to original volume with 0.1 M Veronal. This fraction represents a seven-fold purification and contained the bulk of the activity present in the crude homogenate. The enzyme solution at this stage of purification is stable indefinitely at refrigerator temperatures.

Crystallization of this enzyme has been completed by Dr. Francis Binkley and will be report separately.

Methods of Analysis. In all measurements reported here of the activity of the enzyme glutaminase in the hydrolysis of glutathione the method of Sullivan and Hess (31) was used. This method measures both cysteinylglycine and cysteine. However, since the work to be reported here is concerned only with the first step in

the hydrolysis of glutathione, the activity reported can be considered to be the amount of cysteinylglycine formed.

In all measurements reported here of the hydrolytic products of glutamine formed by this enzyme a modification of Archibald's (1) aeration method was used. The aeration buffer and aeration apparatus were used as described by Archiblad. By our method 3.0 ml. of the aeration buffer were incubated with 2.0 ml. of a trichloroacetic acid filtrate of the digestion mixtures and the ammonia released aerated into 10.0 ml of 0.01 N sulfuric acid. Color was developed by the addition of 3.0 ml of Nessler's solution after a period of 30 minutes. Activity reported is the amount of nitrogen formed as ammonia by glutaminase.

All measurements reported here were made with a Coleman junior spectrophotometer, Model 6 A.

Procedure. The following routine was followed for all work reported here unless otherwise specified. To 5.0 ml of 0.1 M buffer were added 1.0 ml of the enzyme preparation, 1.0 ml of 0.1 M magnesium chloride, 1.0 ml of substrate (the concentrations of glutathione and glutamine will be specified in each case) and water to make a total of 9.5 ml. Metal ions and other materials were dissolved in water, and added in lieu of water.

All digestion mixtures were incubated at 37° for 60 minutes. Hydrolysis was stopped by the addition of 0.5 ml of 50% trichloroacetic acid, and the solution filtered. The filtrate was used immediately in

all analysis.

I Identification of glutaminase and the enzyme cleaving the gamma glutamyl bond of glutathione as the same enzyme.

Krebs (20) reported in 1935 that there was present in the liver, kidney, brain and retina an enzyme that was capable of hydrolyzing glutamine to glutamic acid and ammonia. Archibald (1), in 1944, further described the enzyme glutaminase and developed an enzymatic method for its measurement involving the determination of the ammonia released by the action of glutaminase. The enzymatic hydrolysis of glutathione by tissues of the rat was first reported by Woodward and co-workers (34) in 1935. In 1948 Binkley and Nakamura (4) reported that the first step in the enzymatic hydrolysis of glutathione was the cleavage of the gamma glutamyl bond.

The data to be reported here identifies the enzyme responsible for the hydrolysis of glutamine to glutamic acid and ammonia as the same enzyme which cleaves the gamma glutamyl bond of glutathione.

The steps in the purification of the enzyme have been described earlier. A comparison of the hydrolytic activity of glutaminase against glutamine and glutathione through the steps of purification demonstrates the identity of this enzyme. (Table I) and Figure I).

The rate of hydrolysis of glutathione and glutamine individually by glutaminase has been compared and the kinetics of these reactions are shown in Table II (the figures in Table II are taken from Figure I). The effect of the presence in digestion mixtures

of one of these substrates on the hydrolysis of the other by the enzyme will be described.

Table I

Activity of Glutaminase with Purification

	Substrate:	Glutamine	Glutathione
Enzyme Preparation	mg. N per ml.	Nitrogen formed as NH_3	Cysteinylglycine formed
		A x 10^{-4}mmols per minute	A x 10^{-4}mmols per minute
I	1.2	2.8	2.7
II	0.6	4.3	3.9
III	0.4	4.4	4.0

Enzyme preparations:
I---dialyzed homogenate
II---acid precipitated fraction
III---alcohol (0.3) precipitated fraction

Table II

Hydrolysis of Glutamine and Glutathione by Glutaminase

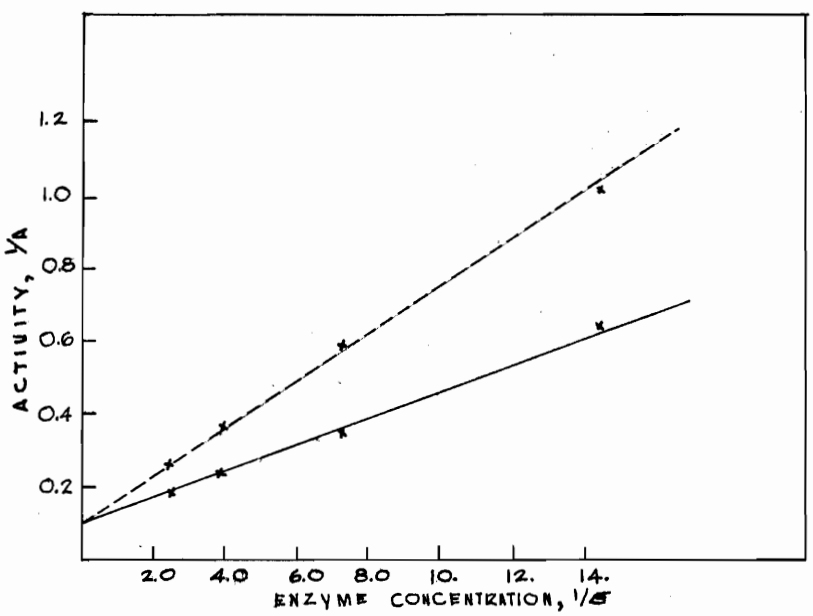
Enzyme Varied Studies

Substrate	Meq./liter	V. max	1/Vmax	K_s/Vmax.
Glutamine	10	0.15	6.67	3.04×10^{-2}
Glutathione	5	0.15	6.67	6.08×10^{-2}

V = activity

Identification of Glutaminase and the Enzyme Which Cleaves the Gamma Glutamyl Bond of Glutathione as the Same Enzyme. Digestion mixtures of the enzyme solutions were in 0.1M barbiturate buffer pH 7.5. The time of incubation was 60 minutes at 37 degrees. 1.02×10^{-2}mmols of glutamine were present in all experiments, and 4.90×10^{-2}mmols of glutathione were present in those experiments showing cysteinylglycine formed. **Figure I.** **Glutaminase activity with substrates glutamine and glutathione. The upper broken line represents cysteinylglycine formed from glutathione, and the lower solid line represents the ammonia formed from glutamine**

Fig. I.



GLUTAMINASE ACTIVITY WITH SUBSTRATES GLUTAMINE AND GLUTATHIONE

by glutaminase. Activity is expressed in mmols x 10^{-4} formed per minute, and enzyme is expressed as mg. of nitrogen contained in digests.

Effect of glutathione on activity of the enzyme, with glutamine as substrate. Since one enzyme of the kidney hydrolyzes two compounds which are in as high a concentration in the tissues of the kidney as are glutathione and glutamine, it is possible that their physiological roles may be in someway associated. To further substantiate the identity of the enzyme, glutaminase, and to study the interaction of these two substrates, experiments were run to illustrate the effect of glutathione on the hydrolysis of glutamine by glutaminase. The activation of the hydrolysis of glutathione, by glutamine, has been described earlier.

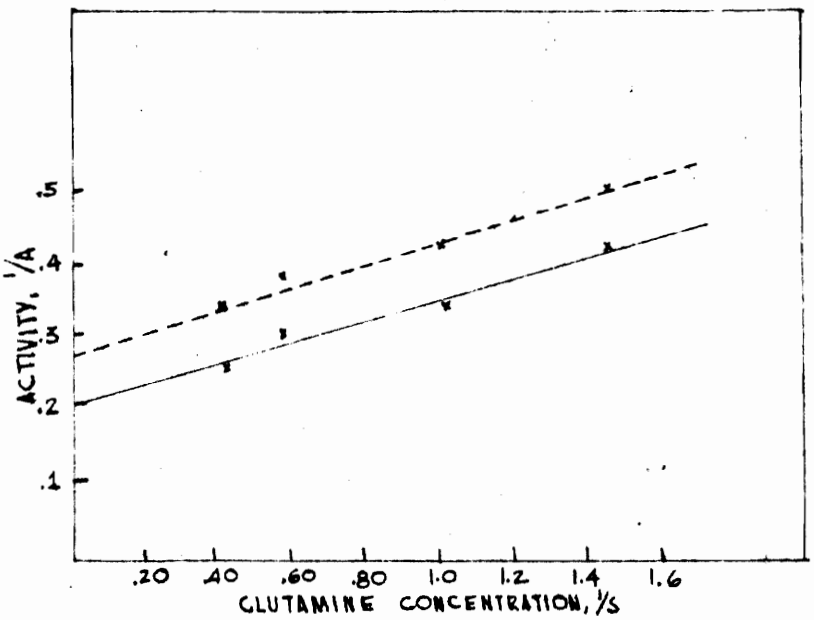
All digests were in 0.1 M barbiturate buffer pH 7.5, with the acid precipitated enzyme preparation. The time of incubation was 60 minutes at 37°. In all digests the molar concentration of glutathione was twice that of glutamine.

Activation of glutaminase, by glutamine, with glutathione as substrate. Hydrolysis of glutathione by glutaminase requires trace amounts of glutamine to be present in digestion mixtures. The activation of glutaminase by glutamine suggests that glutathione is a cosubstrate for glutaminase and, as such, is a mechanism by which the enzyme assures itself of a constant supply of glutamine and thereby a readily available source of urinary ammonia.

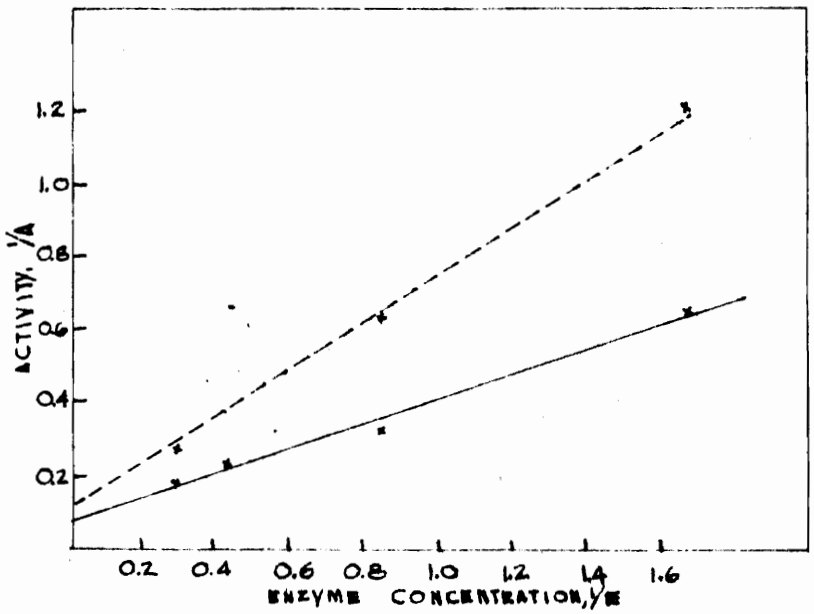
The following graphs illustrate the activation of glutaminase by glutamine in digestion mixtures with glutathione present as the substrate. The acid precipitated enzyme preparation was used in these

Figure II. Glutathione effect on glutaminase with glutamine as substrate. The upper broken line represents the ammonia formed from glutamine by glutaminase in digests containing 0.49 x 10^{-3}mmols of glutathione, and the lower solid line represents the ammonia produced by glutaminase from glutamine in digests not containing glutathione. Glutamine concentration is expressed in mmols x 10^{-3}. Activity is expressed as mmols x 10^{-4} of ammonia formed per minute.

Figure III. Glutamine activation of glutaminase with glutathione as substrate. The upper broken line represents the cysteinylglycine formed in the absence of glutamine, and the lower solid line represents cysteinylglycine formed in digests containing 0.97 x 10^{-3}mmols of glutamine. Activity is expressed as mmols x 10^{-4} of cysteinylglycine formed per minute. Enzyme is expressed as mg. of nitrogen contained in digests.



GLUTATHIONE EFFECT ON GLUTAMINASE
WITH GLUTAMINE AS SUBSTRATE
ENZYME CONCENTRATION CONSTANT



GLUTAMINE ACTIVATION OF GLUTAMINASE
WITH GLUTATHIONE AS SUBSTRATE
GLUTATHIONE CONCENTRATION CONSTANT

studies. The time of incubation was 60 minutes at 37 degrees. 4.09 x 10^{-2}mmols of glutathione were present in all digests.

II Inhibitors of Glutaminase

Inhibitory effect of bromsulfalein. Inhibition of the enzyme glutaminase by bromsulfalein was originally reported by Archibald in 1944. The data presented here further identifies glutaminase as the enzyme which hydrolyzes the gamma glutamyl bond of glutathione. Bromsulfalein acts as a competitive inhibitor of the hydrolytic action of glutaminase in digestion mixtures in which glutathione is present as the substrate, but addition of glutamine to these digestion mixtures relieves the inhibitory effect of bromsulfalein.

Archibald listed the compounds p-benzoquinone and atabrine as inhibitors of glutaminase. It is believed that these compounds will show the same order of inhibition of glutaminase in digestion mixtures in which glutathione was present as the substrate, as that shown by bromsulfalein, and that glutamine in these digests will relieve their inhibitory effects. From the inhibition studies described here, and those earlier reported by Archibald, it appears that inhibition of the enzyme glutaminase is in no way specific for one type of compound, and it should be possible to substantiate the claim that any substance now believed to be excreted by the tubules of the kidney will demonstrate an apparent inhibitory effect of glutaminase.

Inhibition studies were carried out in digests of 0.1M barbiturate buffer pH 8.0 with the acid precipitated enzyme preparation.

Fig. 4.

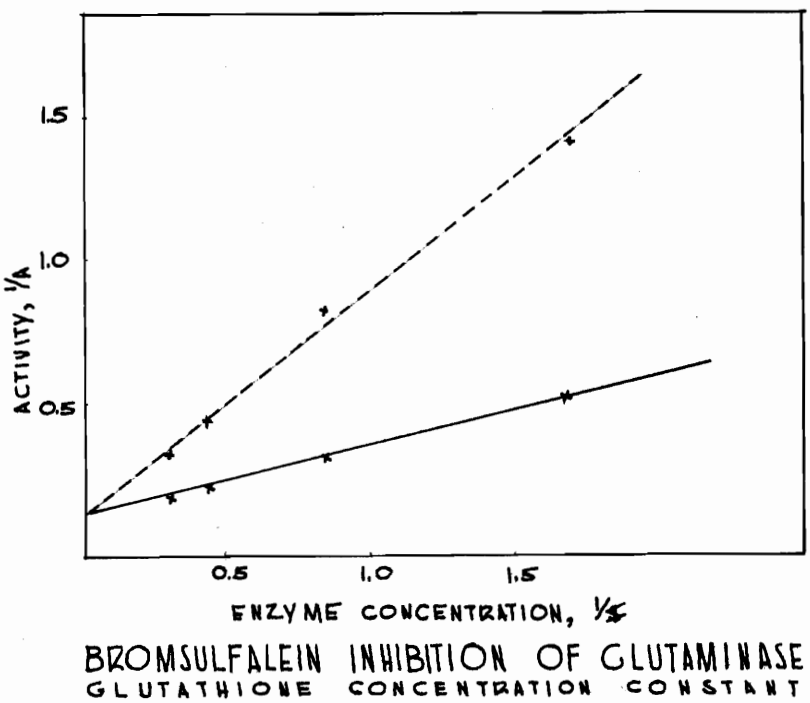
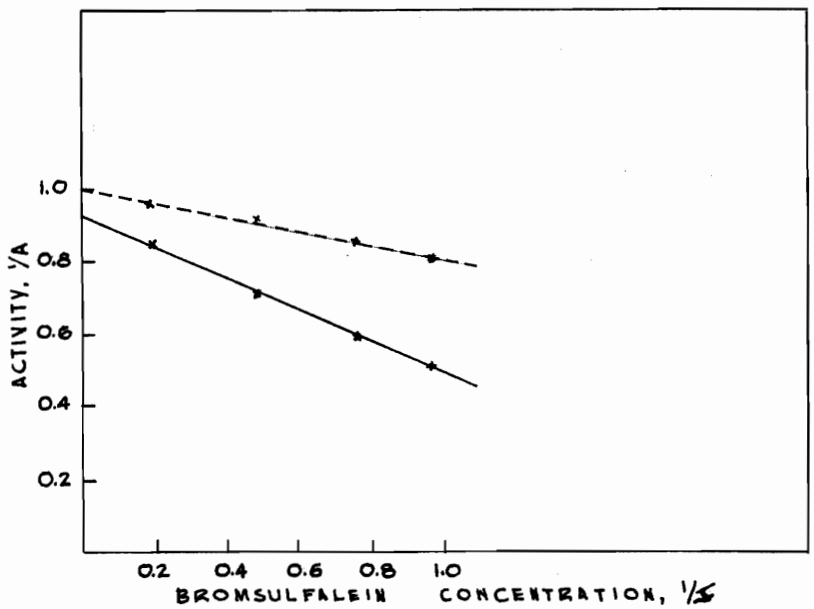


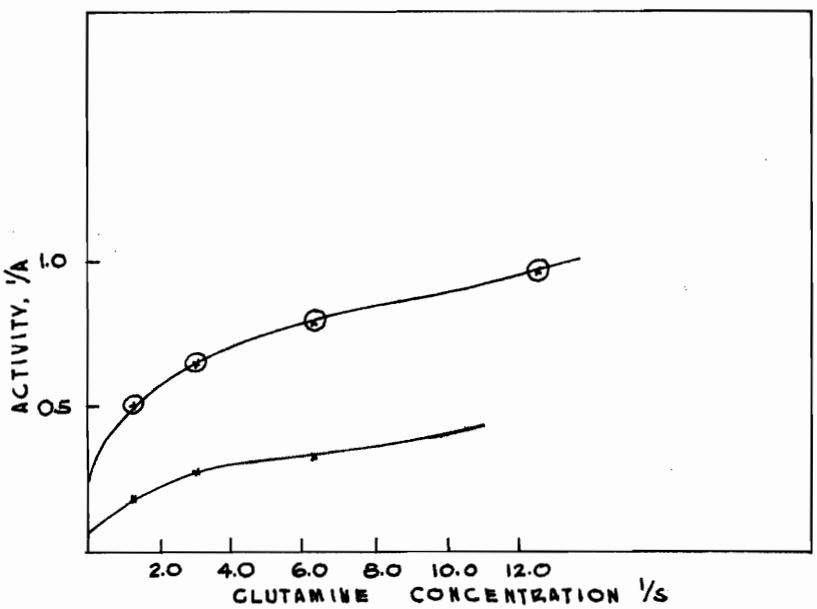
Figure 4. Bromsulfalein inhibition of glutaminase. The upper broken line represents the cysteinylglycine formed in digests containing 2.07 x 10^{-3} mmols of bromsulfalein, and the solid lower line represents the cysteinylglycine formed in digests not containing bromsulfalein. Activity is expressed in mmols x 10^{-4} formed per minute, and enzyme is expressed as mg. of nitrogen contained in digests.

Fig. 5.



BROMSULFALEIN INHIBITION OF GLUTAMINASE
ENZYME AND GLUTATHIONE CONCENTRATION CONSTANT

Fig. 6.



EFFECT OF GLUTAMINE ON
BROMSULFALEIN INHIBITION OF GLUTAMINASE
ENZYME AND GLUTATHIONE CONCENTRATION CONSTANT

Time of incubation was 60 minutes at 37°. Figure 5. The effect of glutamine on bromsulfalein inhibition of glutaminase. The formation of cysteinylglycine from glutathione in the presence of bromsulfalein is shown by the upper broken line, and in the presence of bromsulfalein plus glutamine by the lower line. The glutamine concentration for the lower line was constant at 0.68×10^{-3} mmols per liter. Bromsulfalein concentration is in mmols $\times 10^{-3}$. Figure 6. The effect of glutamine on bromsulfalein inhibition of glutaminase. The formation of cysteinyl-glycine in the presence of 1.03×10^{-3} mmol per liter of bromsulfalein is shown by the upper line and in the absence of bromsulfalein by the lower line. Glutamine concentration is in mmol $\times 10^{-2}$ per liter. In figures 5 and 6 the activity is expressed as the formation of cysteinyl-glycine in mmol $\times 10^{-4}$ per minute.

Inhibitory effect of penicillin. Cavallito (37) has pointed out that if antibiotics inhibit growth by reacting with essential -SH groups in bacterial cells, two explanations for antibiotic action which are possible are (a) that the compounds may react with essential -SH groups of bacterial enzymes; (b) that the compounds may react with the -SH groups in cysteinyl residues, as these are joined at the end of a growing peptide chain during protein anabolism. In his studies of the effect of thiol structures on the rate of inactivation of antibiotics he found that L-cysteinylglycine inactivated penicillin in less than one hour while it required eighteen hours for glutathione to inactivate the antibiotic, and from these studies suggested that penicillin reacted much more readily with compounds containing a free amino group in

the cysteine residue. In our studies penicillin was found to inhibit the cleavage of the gamma glutamyl bond of glutathione by glutaminase. Further study of this inhibition showed that penicillin acts as a competitive inhibitor of glutaminase, and that this inhibition can be overcome by an increase in concentration of the substrate, glutathione or by the presence of glutamine in digestion mixtures.

Preincubation of penicillin with glutathione and with the enzyme individually for periods of two hours failed to show any effect on the inhibition illustrated in digestion mixtures. Apparently during this period of time penicillin does not permanently alter the glutathione or the enzyme molecule, but acts only to block the active surface on the enzyme molecule in its hydrolysis of the substrate glutathione.

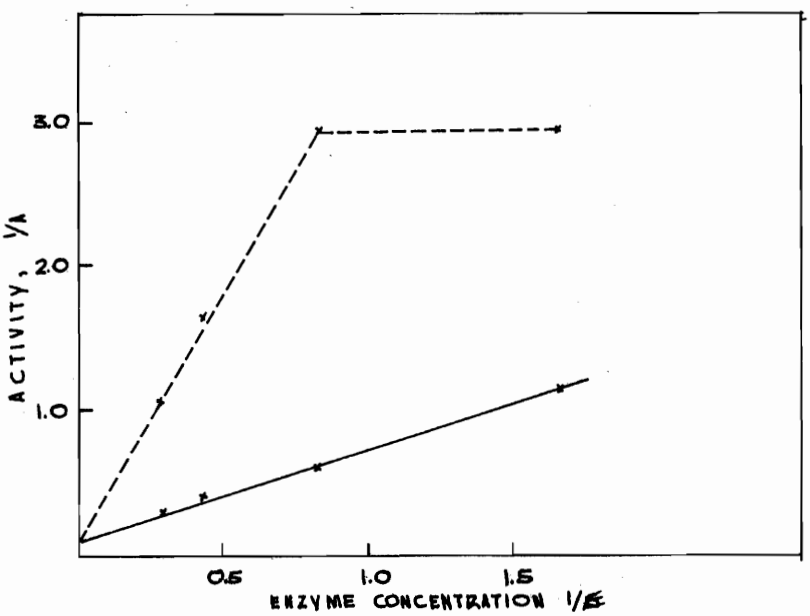
Fischer (38), in 1947, first drew attention to the structural resemblence between glutathione and penicillin. In view of the apparent competitive role of penicillin in digestion mixtures, studies were made of the effect of penicillinase with glutamine and with glutathione as substrates. In all experiments containing 50 units of the dialyzed enzyme, penicillinase (obtained from the Schenley Laboratories), no hydrolytic action was demonstrated against any of the above substrates.

Experiments have been run to demonstrate any effect penicillin might have on the Sullivan method of determination, or on the product of hydrolysis, cysteinylglycine, which Cavallito found rapidly reacted with penicillin. Determination of known concentrations of cysteine, in the presence and in the absence of penicillin, indicate that the inhibition of glutaminase by penicillin is not, in effect, an interference with the method of determination, or with cysteine itself.

Figure 7. Penicillin inhibition of glutaminase. The formation of cysteinylglycine from glutathione in the presence of penicillin is shown by the upper broken line, and in the absence of penicillin by the lower solid line. Activity is expressed as the formation of cysteinylglycine in mmol x 10^{-4} per minute. Enzyme is expressed as mg. of nitrogen contained in digests.

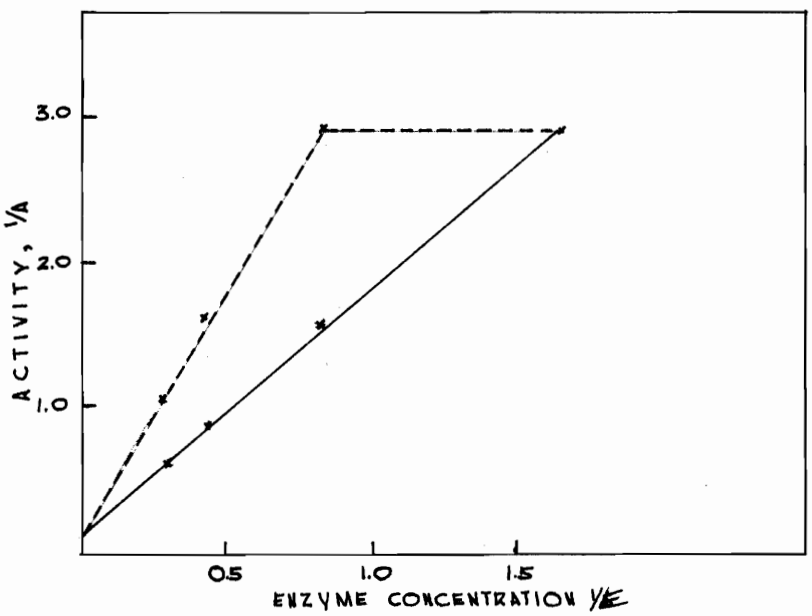
Figure 8. Effect of glutamine on penicillin inhibition of glutaminase. The upper broken line is the same as figure 7 and shows the inhibited formation of cysteinylglycine from glutathione in the presence of penicillin. The lower solid line shows the relief of this inhibition by addition of 1.02 x 10^{-2}mmols of glutamine to the digest. Activity is expressed as the formation of cysteinylglycine in mmol x 10^{-4} per minute. Enzyme is expressed as mg. of nitrogen contained in digests. All digests were in 0.1M barbiturate buffer, pH 8.0. The time of incubation was 60 minutes at 37°. 8.15 x 10^{-2}mmols of glutathione and 8.55 x 10^{-2}mmol of penicillin were present in these experiments.

Fig. 7.



PENICILLIN INHIBITION OF GLUTAMINASE
GLUTATHIONE CONCENTRATION CONSTANT

Fig. 8.



EFFECT OF GLUTAMINE ON
PENICILLIN INHIBITION OF GLUTAMINASE
GLUTATHIONE AND GLUTAMINE CONCENTRATION CONSTANT

Table III

Penicillin Inhibition of Glutaminase

Kinetic Relationships of Penicillin Inhibition of Glutaminase and Relief of This Inhibition by Glutamine

Enzyme Varied Studies

Digestion Mixtures	Km	1/V max	V max
I--Glutathione alone	0.64	0.11	9.1
II-Glutathione + Glutamine	0.38	0.07	14.3
III-Glutathione + Glutamine + Penicillin	1.75	0.07	14.3

These figures taken from Figures VII and VIII

III Effect of Ionized Materials on Glutaminase

In Nakamura's studies of the effects of various ions on the enzyme that hydrolyzed the gamma glutamyl bond of glutathione, he reported that "when measurements were made with higher concentrations of substrate, it was found that at levels of substrate near or greater than saturation additional substrate was inhibitory. The inhibition by higher concentration of substrate was largely abolished by the addition of magnesium ions." (25b). This was the only valid indication for the participation of an inorganic ion in the system. Studies of the effects of various ions on glutaminase reported here indicate that the primary function of this enzyme in the kidney may be in the process of reabsorption carried out

by the tubules of the kidney. These studies have been handicapped by the insolubility of the enzyme. It should be pointed out that all ion effect work was of necessity carried out with enzyme preparations dissolved in 0.1 M sodium barbiturate, and that all digestion mixtures contained 3.0 ml. of a 0.1 M Veronal buffer pH 7.5. For in vitro studies to accurately describe the effect ionized materials are exerting on glutaminase, buffer systems for the individual ions must be worked out in the pH range of 10.

It is realized that the data here described and illustrated by the accompanying graphs is imcomplete in the sense that to correctly describe the effect that ionized materials are exerting on the enzyme glutaminase, a much wider variety of ion concentrations, and thereby more points on these graphs, are needed. The data presented here does show that ionized materials affect glutaminase in two ways. Of the ions so far studied, those ions that are reabsorbed by the kidney tubule from the glomerular filtrate, activate glutaminase when present in digestion mixtures in concentrations normally found in the blood plasma, and in higher concentrations inhibit the enzyme. The incompleteness of the data and graphs presented here lies in the fact that the narrow zone of activation, followed by a zone of inhibition, is not definitely defined.

Effect of ammonium ions on glutaminase. As earlier described, purification of glutaminase was held up until it was realized that following overnight dialysis of the crude homogenate, glutamine was required to activate the enzyme in digestion mixtures in which gluta-

thione was present as the substrate. The rate of hydrolysis of glutathione by purified glutaminase is proportional to the concentration of enzyme, and to the concentration of ammonium ions present in the digest. It is suggested that the hydrolysis carried out by glutaminase involves the breakdown of glutathione in the presence of ammonium ions to cysteinylglycine and glutamine, and that the glutamine formed is further hydrolyzed to glutamic acid and ammonia.

Table IV

Effect of Ions on the Hydrolysis of Glutathione

Expressed as Per cent of Activity of Digests not Containing Additional Ionized Material

Chlorides

M eq. per liter	Lithium	Sodium	Potassium
50	81	101	109
100	71	84	86
500	48	66	52

Sulfates

M eq. per liter	Lithium	Sodium	Potassium
50	91	101	105
100	88	109	111
500	84	88	90

Bicarbonates

M eq. per liter	Lithium	Sodium	Potassium
50	101	103	112
100	84	84	89
500	62	21	11

Fig. 9.

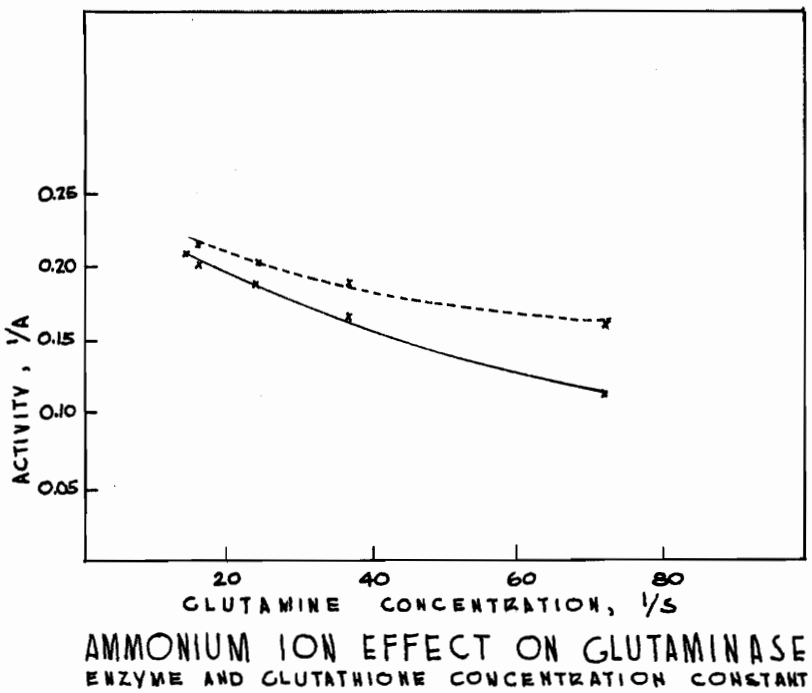
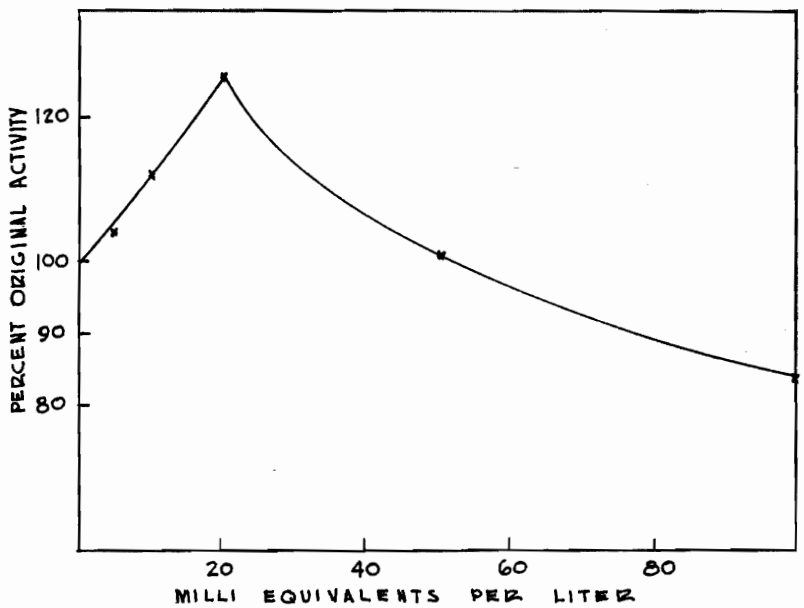


Figure 9. Ammonium ion effect on glutaminase. The upper broken line represents cysteinylglycine formed in digests not containing ammonium chloride ions, and the solid lower line shows the increased amount of cysteinylglycine formed in digests containing 0.61×10^{-4} mmols of ammonium chloride ions. 4.09×10^{-2} mmols of glutathione were present in digestion mixtures buffered with 3.0 ml. of 0.1M veronal buffer, pH 8.1. Activity is expressed in mmols $\times 10^{-4}$ formed per minute, and the concentration of glutamine is expressed as mmols $\times 10^{-2}$ present in digests.

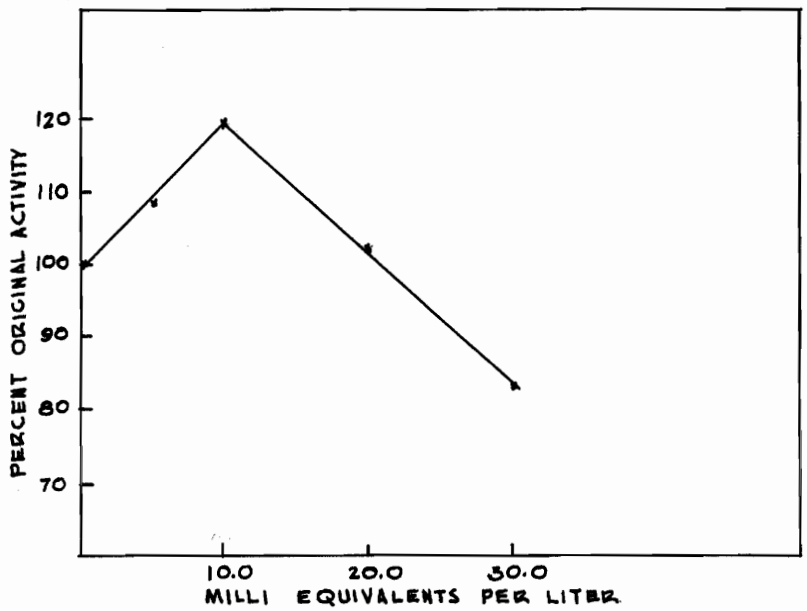
Fig. 10.



EFFECT OF SODIUM BICARBONATE ON GLUTAMINASE

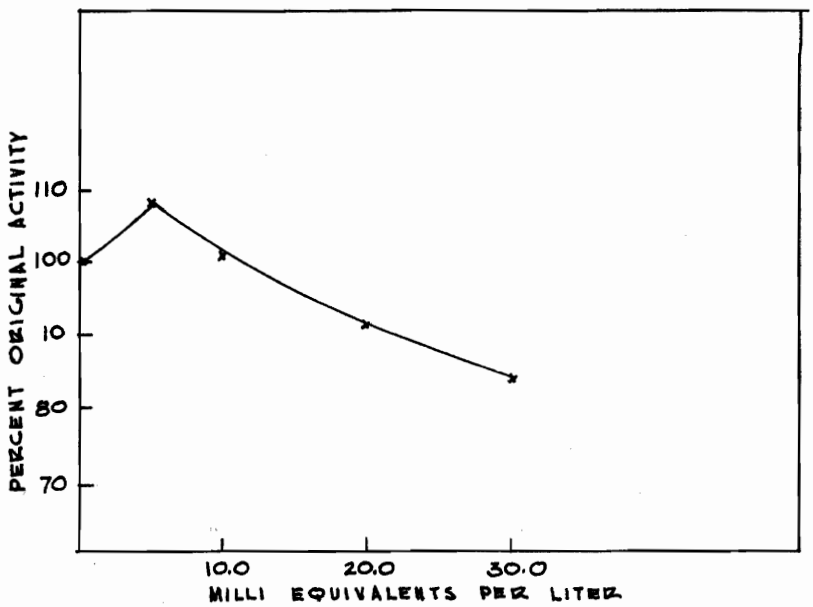
Figures 10, 11, 12, 13, and 14. All ion effect work described in these figures was carried out with the acid precipitated enzyme preparation. All digests contained 4.09×10^{-2} mmols of glutathione, 0.51×10^{-2} mmols of glutamine, and were buffered with 3.0 ml. of 0.1M veronal buffer, pH 7.5. Activity is expressed as per cent of activity found in digests not containing additional ions. Concentrations of ions added to these digests are expressed in milli-equivalents per liter.

Fig. 11.



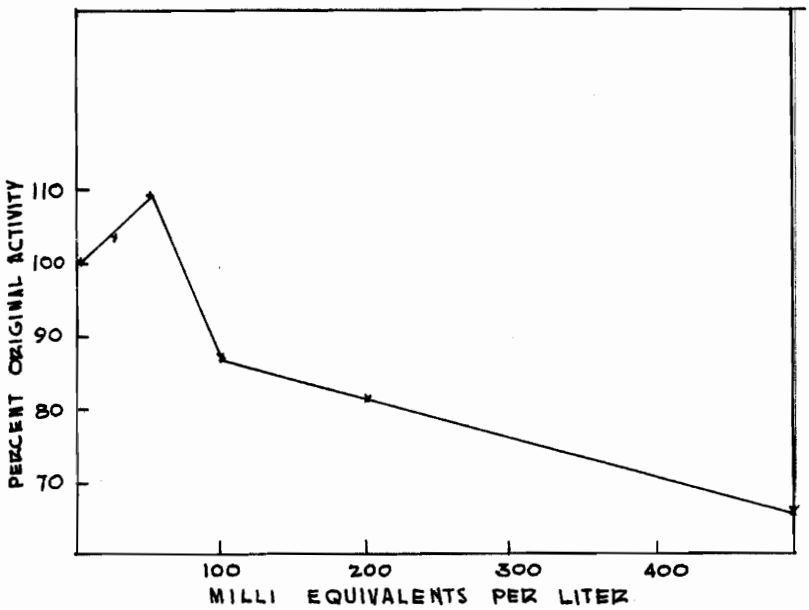
MAGNESIUM CHLORIDE EFFECT ON GLUTAMINASE

Fig. 12.



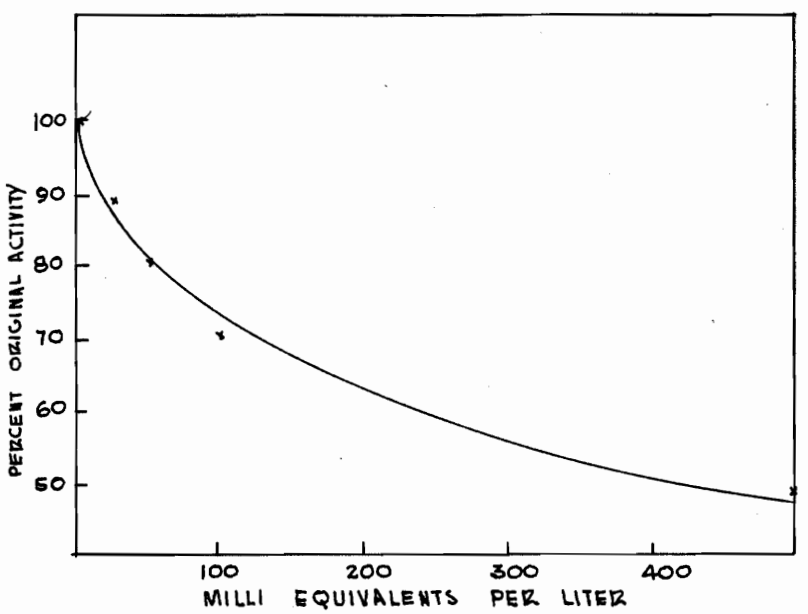
SODIUM PHOSPHATE EFFECT ON GLUTAMINASE

Fig. 13.



POTASSIUM CHLORIDE EFFECT ON GLUTAMINASE

Fig. 14.



LITHIUM CHLORIDE EFFECT ON GLUTAMINASE

Magnesium ions. As demonstrated by the accompanying graph, magnesium ions, when present in concentration up to and slightly greater than those normally found in the blood, activate the hydrolytic action of the enzyme and are inhibitory in greater concentrations.

Bicarbonate ions. In experiments increasing the concentrations of sodium bicarbonate, potassium bicarbonate, and lithium bicarbonate, brought to approximately pH 8, the same type of activation, followed by inhibition, is again apparent. Activation by the bicarbonate ions is correlated with the concentration of these ions in the blood.

Citrate and Sulfate ions. In all digests and at all concentrations within the physiological range tested citrate and sulfate ions were without effect.

Sodium, Potassium, Chloride, and Phosphate ions. Studies with these ions further demonstrated the definite areas of activation, with sharp lines of inhibition, found with other ions. Other than with sodium ions, inhibition by these ions was found in digests in which they were in concentrations greater than those normally present in the renal blood supply. It is felt that the sodium activation area would have shifted to the right on the graph below if a buffer containing no sodium ions had been used in digestion mixtures.

Lithium ions. Various concentrations of an ion not reabsorbed by the tubules of the kidney were found to be inhibitory in digestion mixtures. Thus, at all concentrations tested, lithium sulfate, chloride, and bicarbonate inhibited the action of glutaminase.

DISCUSSION

Previous research on glutathione has awarded many possible functions to this tripeptide, but the q uestion of why this compound is present in so high a concentration in the body tissues and blood has overshadowed all the menial tasks so far ascribed to it. The possibility that in its hydrolysis glutathione served as an integral part of some physiological meghanism was realized by Binkley and Nakamura (4). They knew that one enzyme, abundantly and almost exclusively found in the kidney, carried out the first act of hydrolysis, and that cleavage of the dipeptide bond was accomplished by a different enzyme which did not show this definite specificity for site of action. Thus, if the hypothesis of Binkley and Nakamura was correct, the role of glutathione might well be expected to be a related and integral function of the tissues of the kidney.

Until the enzyme which carries out the first step in the hydrolysis of glutathione could be obtained in a purified form, the relationship of the two circulating gamma glutamyl compounds of the blood, glutamine and glutathione, was not realized. The loss of an activating substance in all attempts at isolation led both Krebs (20) and Archibald (1) to believe that the enzyme which they designated "glutaminase" was highly insoluble, and in fact, was of many types. Loss of activity of enzyme solutions by dialysis alone misled Neubeck and Smythe (26) into reporting the necessity of a coenzyme in the system. Recognition that glutamine was the activating substance made possible the isolation of this enzyme, and thereby the preceding studies. During studies to describe

the activating effect of glutamine, it became apparent that, as glutamine added something to the system, it was itself being altered by the system, independently of the presence or absence of glutathione. Comparison of rate of hydrolysis of two substrates, glutathione and glutamine, by one enzyme, identified glutaminase. Purification had given the enzyme to which Van Slyke, et al, (36) had ascribed the role of formation of urinary ammonia, and the same enzyme cleaved the gamma glutamyl bond of glutathione.

The enzyme is a protein which exhibits strongly acidic properties, is soluble only in the range of pH 11, and apparently functions as a cation exchange resin in the cells lining the kidney tubules. Glutaminase must be assumed to be a functional enzyme of the kidney, in view of the fact that purification represents nearly ten percent of the total protein of this organ.

Activation effects comparable to those shown by glutamine were found in the presence of trace amounts of ammonium ions, which suggests the role glutathione might play in the glutamine-glutaminase system. Apparently the first step in hydrolysis of this tripeptide when ammonium ions are present is the formation of glutamine. Therefore, by this mechanism, glutathione serves as a ready source of glutamine, which is constantly available to the glutaminase for the formation of urinary ammonia, and the large insoluble glutaminase molecule represents an absorptive resin surface which has the power of self-regeneration. From this it would appear that the rate of hydrolysis of glutathione in the intact kidney tissues is proportional to the glutamine concentration present in these tissues.

Further studies of the effect of various ions on this enzyme suggested that glutaminase possibly plays another role in renal physiology. The enzymatic activity of glutaminase is influenced by ionized materials in two ways. For those ions so far studied (and this list now includes nearly all ionized materials normally present in the blood) there is a definite area of concentrations of activation, followed sharply by an area of inhibition. The described studies demonstrate that phosphate, sodium, potassium, magnesium, carbonate, and chloride ions activate the enzyme up to concentrations roughly equivalent to those found in the glomerular filtrate, and in concentrations above this, become proportionately inhibitory. Ions which the body retains by tubular reabsorption, it may be predicted, will activate the enzyme when present in concentrations normally found in the blood. It is significant that sulfate ions, which are not reabsorbed by the kidney from the glomerular filtrate, had no effect on the enzyme. Ions normally not present in the circulating blood, in this case lithium ions, were found to be inhibitory.

It is realized that to describe accurately the effect ionized materials are exerting on this enzyme, and thus to show the role of this enzyme in tubular reabsorption, <u>in vivo</u> experiments are required. The insolubility of the enzyme makes it apparently impossible to work with active enzyme preparations that are not dissolved in buffer systems in the pH range of 11. Labelled ions and animals with explanted kidneys should be extremely useful tools in experiments to understand the mechanisms involved.

Further evidence that glutaminase functions in the tubules of the

kidney as an exchange resin has come from inhibition studies which offer an interpretation of the secretory mechanism now attributed to the cells of the tubules. Penicillin inhibition was shown to be competitive in nature, and in digestion mixtures to have no altering effect on the substrate, glutathione, or the enzyme. Inhibition is inversely proportional to the concentration of the enzyme, and is removed by increasing the substrate. Glutamine quantitatively removed the inhibition. If this is considered a combination of penicillin with the enzyme, the apparent inhibition is perhaps a temporarily saturated absorptive surface or mechanism. Inhibition by a substance bromsulfalein, used to measure kidney and liver clearances and blood flow, showed the same characteristics, and it should be possible to show that substances with renal clearances greater than inulin do inhibit glutaminase. It seems highly possible that such compounds circulating in the blood are picked up by the sulfhydryl groups of glutathione, and in this form transported to the kidney, where they are passed into the tubular fluid by this enzyme exchange medium.

Any comprehensive work in an attempt to describe renal absorption and secretion must describe the hormonal influences, but in view of the present work, it may be presumed that the various effects of hormones now ascribed to function in the kidney are in effect associated with the enzyme, glutaminase.

The concentrations of asparagine and asparaginase in plants suggests a comparable role in plant physiology. If this assumption is correct, the researcher would have available a workable tool for further study of the implications of this theory as applied to the plant world.

SUMMARY

(1) Data identifying the enzyme responsible for the first step in the hydrolysis of glutathione, the cleavage of the gamma glutamyl bond, as the same enzyme responsible for the hydrolysis of glutamine to glutamic acid and ammonia is reported here.

(2) Activation studies have suggested the role glutathione perhaps plays in the glutamine-glutaminase system. The activation of glutaminase by trace amounts of ammonium ions in the hydrolysis of glutathione suggests that the body utilizes the first steps in the hydrolysis of this tripeptide to assure a constant supply of glutamine to the enzyme glutaminase for the formation of urinary ammonia.

(3) Inhibition studies have been described which make possible the assumption that another role of glutathione in the body is that of a transport mechanism by which certain materials are carried to the kidney for excretion.

(4) Studies of the physical properties of glutaminase have suggested that the glutaminase molecule represents a cation exchange resin within the tubules of the kidney. The ion studies described here have shown that glutaminase is influenced by the ionized material of the glomerular filtrate in two ways: at concentrations in which these ions are normally present in the blood, they activate the enzyme, but in higher concentrations these ions are inhibitory. These observations have been extended to form the basis of a theory explaining the mechanism by which the processes of reabsorption and secretion are carried out by

the tubules of the kidney nephron.

BIBLIOGRAPHY

(1) Archibald, R.M. Preparation and assay of glutaminase for glutamine determinations. J. Biol. Chem. 154:657. 1944.

(2) Archibald, R.M. Chemical characteristics and physiological roles of glutamine. Chem. Reviews. 37:161 1945.

(3) Archibald, R.M. Chemical characteristcs and physiological roles of glutamine. II. Euclides. 7:251. 1947.

(4) Binkley, F., and Nakamura, K.K.. Metabolism of glutathione. I. Hydrolysis by tissues of the rat. J. Biol. Chem. 173:411. 1948

(5) Carte, C.E. and Greenstein, J.P.. Acceleration of enzymatic desamination of glutamine by several inorganic ions. J. Natl. Cancer Inst. 7:433. 1947

(6) Chibnall, A.C., and Westall, R.G. Estimation of glutamine in the presence of asparagine. Biochem. J. 26:122. 1932.

(7) Errera, M., and Greenstein, J.P. Desamination of glutamine and aspargine in normal and neoplastic hepatic tissues. J. Natl. Cancer Inst. 7:285. 1947

(8) Errera, M., and Greenstein, J.P. Effects of acidity and of heating on the capacity of rat liver extracts to desaminate glutamine and asparagine. 7:437. 1947.

(9) Geddes, W.F. and Hunter, A. Observations upon the enzyme asparaginase. J. Biol. Chem. 77:197. 1928.

(10) Goncalves, J.M. and Greenstein, J.P. Enzymatic deamidation of glutamine in the presence of pyruvate furnished by concomitant reactions. J. Natl. Cancer Inst. 7:269. 1947.

(11) Goncalves, J.M. and Greenstein, J.P. Desamidation of amino acids in rat liver extracts of varying concentrations. J. Natl. Cancer Inst. 7:281. 1947.

(12) Grassman, Dyckerkoff, and Eibeler. The enzymatic cleavage of glutathione. Z. Physiol. Chem. 189:112. 1930. (Original not seen; abstracted in chem. abstract. 24:2200. 1930.)

(13) Grassman, W. and Mayr, O. Yeast asparaginase. Z. Physiol. Chem. 214:185. 1933. (Original not seen; abstracted in chem. abstract. 27:1180. 1933)

(14) Hamilton, P. B. and Tarr, R. R. Glutamine: a major constituent of free alpha amino acids in animal tissues and blood plasma. J. Biol. Chem. 158:397. 1945.

(15) Hamilton, P. B., and Tarr, R. R. Gasometric determination of glutamine amino acid carboxyl nitrogen in plasma and tissue filtrates by the ninhydrin-carbon dioxide method. J. Biol. Chem. 158:375. 1945.

(16) Harington, C. R. and Mead, T. H. Synthesis of glutathione. J. Biol. Chem. 88:409. 1930.

(17) Hopkins, F. G. On an autoxidizable constituent of the cell. J. Biol. Chem. 15:286. 1921.

(18) Kendall, E. C., Mason, H. L. and McKenzie, B. F. A study of glutathione. J. Biol. Chem. 88:409. 1930.

(19) Kendall, E. C., Mason, H. L. and McKenzie, B. F. A study of glutathione. J. Biol. Chem. 87:155. 1930.

(20) Krebs, H. A. Metabolism of amino acids. IV. The synthesis of glutamine from glutamic acid and ammonia and the co-enzymatic hydrolysis of glutamine in animal tissues. Biochem. J. 29:1951. 1935.

(21) Leuthardt, F. A., and Glasson, B. Formation of glycine from serine. Helv. Chim. Acta. 25:245 or 630. 1942.

(22) Lohmann, K. Beitragzur enzmiztischen unwandlung von synthitischen methyl glyonal in milch sauere. Biochem. Z. 254:332. 1932. (Original not seen: abstracted 117 chem. abstract. 27:317. 1932.)

(23) Mason, H. L. A study of glutathione. J. Biol. Chem. 90:25. 1931.

(24) McIlwain, H. Metabolism and functioning of vitamin-like compounds III. Products of the decomposition of glutamine during streptococcol glycolysis. Biochem. J. 40:460. 1946.

(25)a Nakamura, K. K., and Binkley, F. Colorimetric estimation of cysteine. J. Biol. Chem. 173:407. 1948.

(25)b Nakamura, K. K. The enzymatic hydrolysis of glutathione by the tissues of the rat. Unplublished M. S. Thesis. Salt Lake City, Utah, University of Utah Library. 1948.

(26) Neubeck, C. E. and Smythe, C. V. Glutamine hydrolysis by guinea pig. Arch. Biochem. 4:465. 1944.

(27) Price, V. E., and Greenstein, J. P. Studies on the effect of pyruvate on the deamidation of glutathione, asparagine and related compounds, J. Natl. Cancer Inst. 7:275. 1947.

(28) Schroeder, E. F., Munro, M. P. and Weil L. The enzymatic nature of kidney antiglyoxalase. J. Biol. Chem. 110:181. 1935.

(29) Schroeder, E. F. and Woodward, G. E. A titrimetric modification of the glyoxalase method for the estimation of reduced glutathione. J. Biol. Chem. 129:283. 1939.

(30) Schroeder, E. F. and Woodward, G. E. The enzymatic hydrolysis of glutamine by rat kidney. J. Biol. Chem. 120:209. 1935.

(31) Sullivan, M. X. and Hess, W. C. Improvements in methods of hydrolysis of protein. J. Biol. Chem. 117:423. 1937.

(32) Woodward, G. E. Glyoxase as a reagent for the microdetermination of glutamine. J. Biol. Chem. 109:1. 1935.

(33) Woodward, G. E. and Fry, B. Determination of glutamine. J. Biol. Chem. 97:465. 1935.

(34) Woodward, G. E., Munro, M. P. and Schroeder, E. F. The antigyoxalase action of kidney and pancreas preparations. J. Biol. Chem. 109:11. 1935.

(35) Woodward, G. E. and Reinhart, F. E. The effect of pH on the formation of pyrrolidonecarboxylic acid during the enzymatic hydrolysis of glutathione by rat kidney extract. J. Biol. Chem. 145:605. 1942.

(36) Van Slyke, D. D., Phillips, R. A., Hamilton, P. B., Archibald, R. M., Futcher, P. H., Hiller, A. Glutamine as source material of urinary ammonia. J. Biol. Chem. 150:481. 1943.

(37) Cavallito, Chester J. Relationship of thiol structures to reaction with antibiotics. J. Biol. Chem. 164:29. 1946.

(38) Fischer, E. Comments by readers. Science. 106:147. 1947.

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